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A Hybrid Toxin from Bacteriophage f1 Attachment Protein and Colicin E3 Has Altered Cell Receptor Specificity

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A hybrid protein was constructed in vitro which consists of the first 372 amino acids of the attachment (gene III) protein of filamentous bacteriophage f1 fused, in frame, to the carboxy-terminal catalytic domain of colicin E3. The hybrid toxin killed cells that had the F-pilus receptor for phage f1 but not F^- cells. The activity of the hybrid protein was not dependent upon the presence of the colicin E3 receptor, BtuB protein. The killing activity was colicin E3 specific, since F^+ cells expressing the colicin E3 immunity gene were not killed. Entry of the hybrid toxin was also shown to depend on the products of *tolA*, *tolQ*, and *tolR* which are required both for phage f1 infection and for entry of E colicins. TolB protein, which is required for killing by colicin E3, but not for infection by phage f1, was also found to be necessary for the killing activity of the hybrid toxin. The gene III protein-colicin E3 hybrid was released from producing cells into the culture medium, although the colicin E3 lysis protein was not present in those cells. The secretion was shown to depend on the 18-amino-acid-long gene III protein signal sequence. Deletion of amino acids 3 to 18 of the gene III moiety of the hybrid protein resulted in active toxin, which remained inside producing cells unless it was mechanically released.

The protein antibiotic colicin E3 kills susceptible *Escherichia coli* by specifically cleaving its 16S ribosomal RNA (8, 9, 20). To reach its cytoplasmic target, colicin must cross both the outer and inner membranes of sensitive cells. The initial stage in the entry of susceptible cells by colicin involves its binding to specific receptors on the outer membrane. For colicin E3, as for all of the E colicins and colicin A, the receptor protein is the vitamin B_{12} receptor, the product of *btuB* (17, 35). The steps subsequent to binding are less well understood. After binding to the receptor, the colicin may interact with other bacterial membrane proteins during its translocation across both membranes to the cytoplasm. Mutations in *tolA*, *tolB*, *tolQ*, and *tolR* (18, 37, 38), and possibly in other genes (10), render cells insensitive to the colicin without impairing the initial binding step, presumably by blocking transfer across one or both membranes. Such tolerant mutants form mucoid colonies and have a number of other phenotypes typical of outer membrane defects, such as sensitivity to a variety of antibiotics and detergents (6).

Protease digestion experiments with the 60-kilodalton colicin E3 and with the closely related cloacin DF13 (also an RNase) have provided evidence for a tripartite domain structure (12, 25, 33, 40). The catalytic domain has been localized to the carboxy terminus; a proteolytic fragment consisting of the carboxy-terminal one-sixth of colicin E3 cleaves ribosomes in vitro. This portion of the colicin also contains the binding site for the E3 immunity protein, which protects colicinogenic cells from the lethal effects of the specific colicin they produce. The receptor-binding function maps to the central portion of colicin E3, and the amino-terminal portion is thought to participate in translocation of the colicin into the cell.

Many of the *tol* mutations that block colicin entry also render cells resistant to infection by the filamentous bacteriophage f1 (M13 or fd). Mutations in *tolA* (37), *tolQ*, or *tolR* (38, 39), but not *tolB*, block infection without blocking adsorption of the phage to its receptor, the F pilus. f1 faces much the same problem of entry as does colicin. In order to deposit its genome in the cytoplasm, it, too, must cross both the outer and inner membranes of the cell. The fact that both the phage and colicin are blocked by a common set of *tol* mutations suggests that the two may have evolved a similar solution to the entry problem.

Binding of phage f1 to its F-pilus receptor has been shown to involve gene III protein, a minor component of the phage virion found exclusively at one end of the particle (43). Experiments using phage with deletions within gene III, as well as protease digestion experiments, have implicated the amino-terminal part of the protein in pilus binding (3, 15, 16, 26, 32).

Newly synthesized gene III protein is transiently integrated into the inner membrane of *E. coli*, where the assembly of phage particles occurs. Mature gene III protein is 406 amino acids long; it is synthesized with an 18-residue signal sequence that is removed as the protein is inserted into the host membrane (4, 31). The protein has, near its carboxy terminus, a hydrophobic region of 23 consecutive, uncharged amino acids which serve as a membrane anchor. Removal of that region converts gene III protein from an integral membrane protein to a secreted one (11).

Gene III protein in a bacterial cell, either synthesized by infecting phage or by the gene III cloned on a multicopy plasmid, confers upon that cell a variety of phenotypes that closely mimic the tolerant phenotype conferred by the *tolA*, *tolQ*, or *tolR* mutation in *E. coli*. Thus, cells synthesizing gene III protein become sensitive to deoxycholate, tolerant to killing by colicins E1, E2, E3, and K, and resistant to infection or superinfection by filamentous phage, and they show leakage of periplasmic proteins (7, 37, 44). Deletions in certain amino-terminal portions of gene III protein no longer confer these phenotypes (7; N. G. Davis, unpublished results).

The entry processes for phage f1 and colicin E3 share certain features, even though they use different cell surface receptors. By fusing coding sequences from the putative f1

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TABLE 1. Bacterial strains

Strain	Relevant characteristics	Construction or immediate source
K38	HfrC Sup$^+$ (λ)	This laboratory
K381	K38 *btuB*	Selected for resistance to colicin E3
W3110	Wild-type *E. coli* K-12, F$^-$	This laboratory
W3110(E3)	Colicin E3 producer	D. Helinski
K361	W3110 *rpsL*	M. Nomura, standard colicin indicator
K561	K38 *lacI*^{q}	This laboratory
K440	F$^+$ *dam-3*	This laboratory
K311	F$^+$ *thr leu lacY minA* T6^s *gal minB thi rpsL*	Minicell producer P678-54 (2)
K1021	K361 λ^r	Selected with λ *vir*
GC2438	F′ *lacI*^{q} *lacZ*::Tn*5*	S. Gottesman (National Institutes of Health)
K1034	W3110 *lacI*^{q} *lacZ*::Tn*5*	P1 transduction from strain GC2438
CGSC6451	F$^+$::Tn*10*	B. Bachmann (Yale University)
CGSC4923	F$^-$ *tolA*	B. Bachmann
CGSC4924	F$^-$ *tolB*	B. Bachmann
CGSC6405	F$^-$ *btuB*::Tn*10*	B. Bachmann
A160	F′::*bla*	M. Russel (this laboratory)
K949	CGSC4923 *tolA btuB*::Tn*10*	P1 transduction from strain CGSC6405
K1017	K949 F′::*bla*	By mating with A160
K1042	CGSC4924 F$^+$ *tolB rpsL*	CGSC4924 selected for *rpsL* on streptomycin, mated with CGSC6451, selected for *rpsL* Tet^r
K1041	K38 *tolQ* (*fii-1*::Tn*10*)	T.-P. Sun (Duke University)
K1089	TPS 300=GMI*tolR*::Cm^r	T.-P. Sun
K1090	TPS 946=*tolQ* (GMI *fii-6 zbj*-1::Tn*10*)	T.-P. Sun
K1074	K361 F$^+$::Tn*10*	By mating K361 with CGSC6451

adsorption protein, gene III protein, to sequences encoding the catalytic moiety of colicin E3, we have created an active toxin with a changed receptor specificity. Whereas native colicin E3 kills only *btuB*$^+$ cells regardless of the presence or absence of the F pilus, the gene III-E3 hybrid protein kills only F$^+$ cells regardless of the presence or absence of BtuB. Furthermore, this study demonstrates that the gene III protein of filamentous phage is sufficient for both pilus binding and for initiating the transfer of a toxin moiety across *E. coli* membranes to the cytoplasm.

MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. The bacterial strains used in this study are listed in Table 1. Bacteriophage P1 (Cm^r *clr-100*) was a gift from M. Russel (this laboratory). P1 transductions were carried out by the method of Miller (29).

Restriction enzymes were from New England BioLabs, Inc., and Bethesda Research Laboratories; Klenow fragment of DNA polymerase was from Boehringer Mannheim Biochemicals; T4 DNA ligase was from New England BioLabs; polynucleotide kinase was from Pharmacia. The enzymes were used according to the instructions of the manufacturers. Plasmid DNA was purified as described elsewhere (45). Transformations were done according to the method of Maniatis et al. (27).

The construction of plasmid pKSJ5 is diagrammed in Fig. 1. Restriction sites were based on the sequence of Masaki and Ohta (28). ColE3 plasmid DNA grown on K440 (a *dam*$^-$ host) was linearized with *Cla*I. The sticky ends thus generated were filled in with Klenow fragment, and the linear DNA was purified on a 0.6% agarose gel and electroeluted on an IBI electroelution apparatus. Phosphorylated 12-mer *Hin*dIII linkers (no. 18078 from Collaborative Research, Inc.) were ligated to the linearized ColE3 DNA. This linker was selected so that the final fusion generated between gene III and the colicin E3 gene would be in the correct reading frame. The DNA was then cleaved with *Hin*dIII and *Bcl*I. The resulting fragment, containing the colicin E3 enzymatic domain and the entire E3 immunity gene, was ligated between the *Hin*dIII and *Bam*HI sites of pBR322 to give pKSJ4. Cells bearing pKSJ4 were Tet^s and were immune to colicin E3 because they contained an intact gene for the immunity protein. The construction of pND372 is described by Davis et al. (11). That plasmid contains the sequences encoding the first 372 amino acids of phage f1 gene III protein cloned, under *lac* control, between the *Eco*RI and *Hin*dIII sites of pBR322. pKSJ5 was generated by digesting both pKSJ4 and pND372 with *Pst*I and *Hin*dIII and ligating the appropriate fragments, which had been purified on 0.8% agarose gels.

pKSJ17 has been described elsewhere (23). It is ColE3 with a Tn*5* insertion which inactivates its colicin E3 gene but still allows expression of wild-type amounts of E3 immunity protein.

The plasmid pND277 is pKSJ5 with a deletion encompassing the nucleotides encoding amino acids 3 to 18 of the precursor form of gene III protein. It also has a mutation of its second amino acid from lysine to isoleucine. To construct pND277, first plasmid pND262 was constructed from pND372 (Fig. 1), which contains cloned gene III. Site-directed oligonucleotide mutagenesis (14) was used to create a *Bcl*I site overlapping the first, second, and third codons of gene III. This mutation also changes the second amino acid of gene III from lysine to isoleucine. A second mutant gene III plasmid, pND151, was also created by oligonucleotide mutagenesis of pND372; this mutation creates a *Pvu*II site overlapping codons 18, 19, and 20 but does not result in a coding change.

To delete the sequences encoding the signal peptide and to create pND270, pND262 was cut with *Bcl*I and the ends were filled with DNA polymerase I Klenow fragment. The resulting linear DNA was reclosed with T4 DNA ligase, which created a new *Cla*I site where the *Bcl*I site had been. The resulting plasmid was cut with *Cla*I, the sticky ends were filled with Klenow fragment, and the DNA was di-

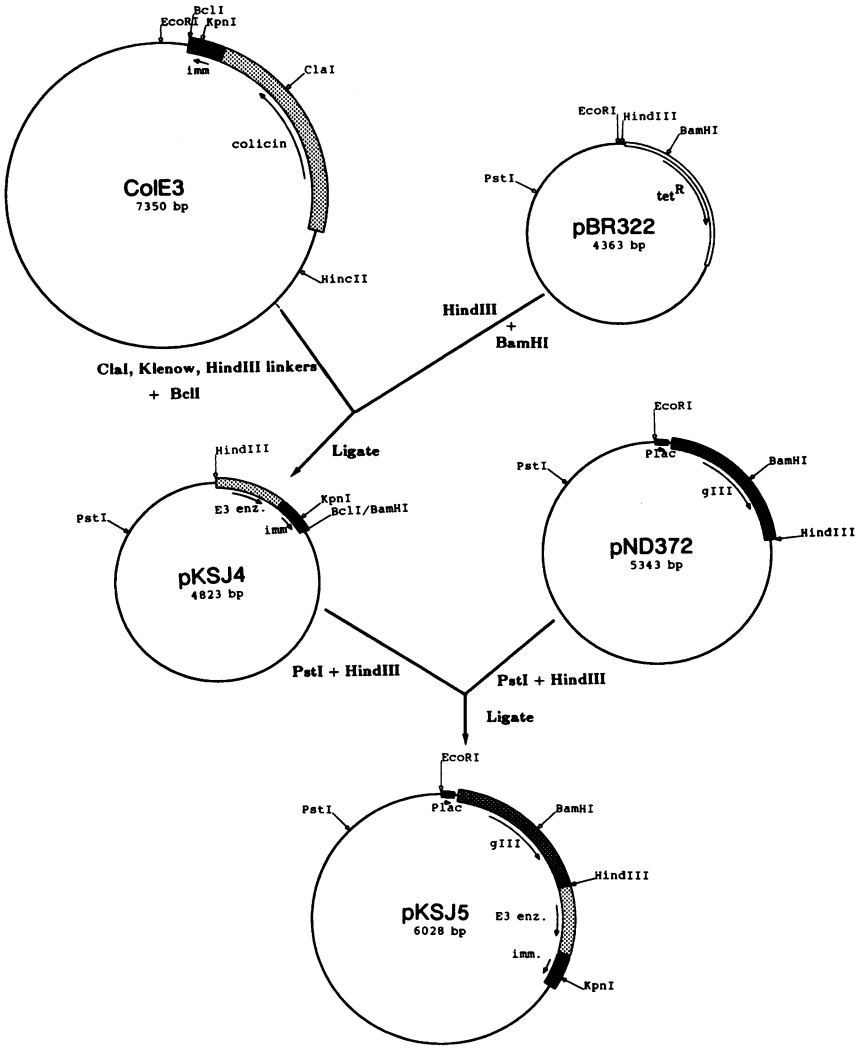

FIG. 1. Construction of pKSJ5 encoding phage f1 gene III-colicin E3 hybrid protein. bp, Base pairs; E3 enz., gene for E3 enzyme; imm., gene for immunity protein.

gested with *Pst*I. The resulting small fragment was ligated to the large fragment resulting from a double digest of pND151 with *Pvu*II and *Pst*I. DNA sequencing confirmed that the resulting plasmid, pND270, is identical to the starting cloned gene III plasmid, except that codons 3 to 18 are deleted. The predicted mutant protein should begin with the sequence formylmethionine$_1$-Ile$_2$-Ala$_{19}$-Glu$_{20}$.

Finally, to introduce the signal sequence deletion into the gene III-colicin E3 gene fusion, the large *Pst*I-*Bam*HI fragment of pKSJ5 (Fig. 1) was ligated to the small *Pst*I-*Bam*HI fragment from pND270 to give pND277.

Colicin and fusion protein assays. Colicin activity, both from intact colicin and from the fusion proteins, was assayed by spotting 20-μl aliquots of the sample to be tested on freshly seeded lawns of the appropriate indicator strain. The indicator cells used generally carried either a chromosomal marker or a plasmid, such as pBR322, which conferred on them an antibiotic resistance not conferred by pND372 or pKSJ5. The lawns were then grown on the appropriate antibiotic-containing plates, so that growth on the plate of the cells being tested for colicinlike activity did not interfere with the assay. Colicin E3 was purified as described elsewhere (19).

In vivo labeling and fractionation of proteins and immune precipitations. Cells to be examined were grown in minimal DO medium (42) without methionine to a density of about 4 × 10^8/ml and then induced with 1 mM isopropyl-β-D-thiogalactoside (IPTG). Fifteen minutes later, 0.2-ml samples were labeled with 10 to 20 μCi of [^{35}S]methionine (1,000 Ci/mmol; New England Nuclear Corp.). After 45 s, the cells were precipitated with cold 5% trichloroacetic acid. Proteins synthesized by minicells, prepared as described previously (34), were labeled in the same way. Immune precipitations were performed as described previously (11). Gene III protein-specific rabbit antiserum was the very generous gift of W. Konigsberg (Yale University). Antiserum against β-lactamase was the gift of Peter Model (this laboratory). Antisera against colicin E3 and E3 immunity protein were prepared as described previously (22). ^{35}S-labeled proteins were analyzed on sodium dodecyl sulfate-polyacrylamide gels (18).

RESULTS

Construction of pKSJ5. The plasmid pKSJ5 (Fig. 1) has the first 372 codons of gene III protein fused, in frame, to a

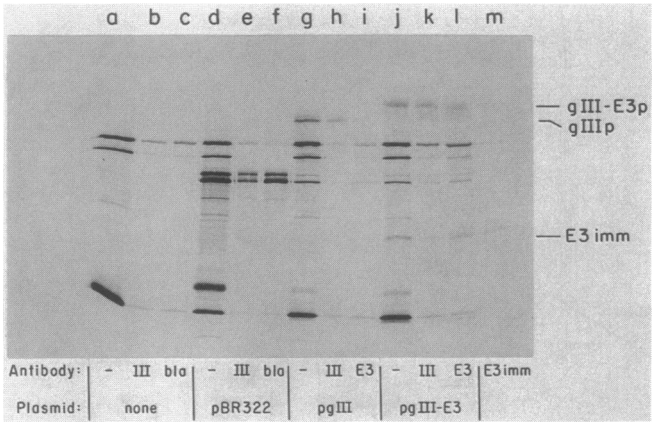

FIG. 2. Proteins synthesized by minicells bearing plasmids encoding gene III protein or gene III-colicin E3 fusion protein. [^{35}S]-methionine-labeled proteins synthesized in minicells were immune precipitated and run on 19% polyacrylamide–sodium dodecyl sulfate gels. Control minicells without plasmid (lanes a to c), with pBR322 (lane d to f), with pND372 (lanes g to i), and with pKSJ5 (lanes j to m) are shown. Antisera against gene III protein (lanes b, e, h, and k), against colicin E3 (lanes i and l), against β-lactamase (lanes c and f), or against colicin E3 immunity protein (lane m) were used to immunoprecipitate the proteins. Total proteins synthesized by the minicells were run in lanes a, d, g, and j. The proteins synthesized by the plasmidless minicells are primarily three very abundant outer membrane proteins whose messenger RNAs have very long half-lives (5). Abbreviations: gIII, gene III; gIIIp, gene III protein; gIII-E3p, gene III-colicin E3 protein; E3 imm, E3 immunity protein; bla, β-lactamase.

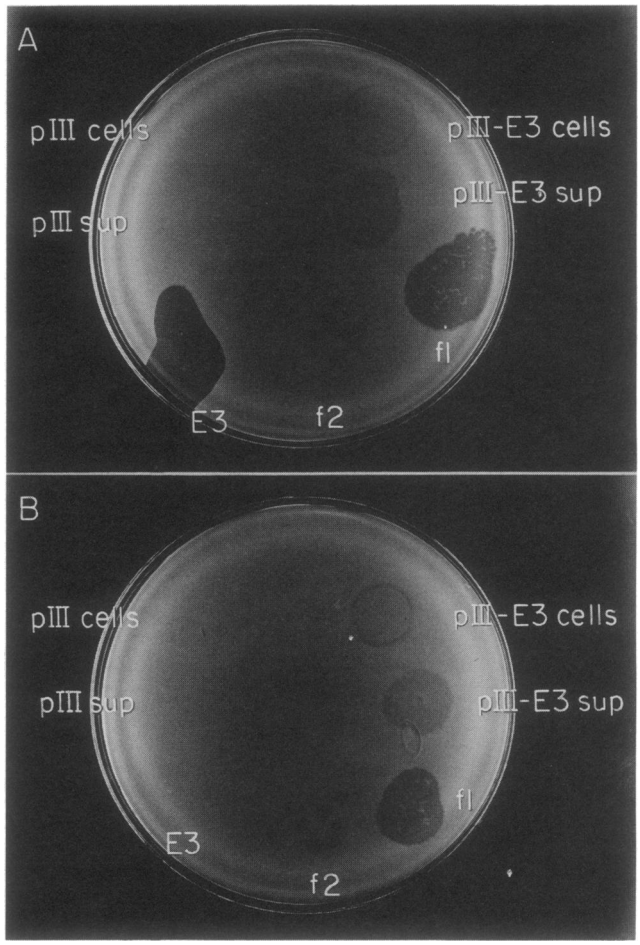

FIG. 3. Killing activity of gene III-colicin E3 fusion protein on F$^+$ *E. coli*. K1034 cells bearing either pND372 (pIII) or pKSJ5 (pIII-E3) were induced for 2 h with IPTG. A 20-μl amount of the cultures (cells) or culture supernatants (sup) was spotted on lawns of indicator bacteria. E3, 0.4 μg of colicin E3 in 20 μl was spotted on the lawns; f1 and f2, 20 μl of appropriate dilutions of these phages were spotted on the indicator lawns. (A) Indicator lawn of F$^+$ bacteria, K38(pBR322); (B) indicator lawn of F$^+$ *btuB* bacteria, K381(pBR322).

796-base-pair portion of ColE3 encoding both the catalytic domain of colicin E3 and the entire E3 immunity protein gene. The gene for the colicin E3 lysis protein (23, 28) is not present in pKSJ5. The fusion protein is inducibly expressed from the *lac* promoter. The gene III parent plasmid to pKSJ5 is pND372, a deletion derivative lacking the carboxy-terminal 52 codons of gene III. This derivative is missing the carboxy-terminal, hydrophobic membrane-spanning domain of gene III protein and, as a result, is secreted across the membrane of the cell (11).

Some of the phenotypes associated with gene III expression were checked in cells bearing pKSJ5. Cells bearing pKSJ5 are immune to colicin E3 but sensitive to colicin E2 when grown on medium lacking IPTG. This immunity results from the presence of the E3 immunity protein, whose synthesis is constitutive in this construct (23, 30). In the presence of IPTG, these cells, like those bearing pND372, become tolerant to both colicin E2 and E3 as a result of the induced expression of the gene III moiety of the fusion protein. These cells also show IPTG-dependent resistance to infection by phage f1.

Proteins synthesized by pKSJ5. Minicells carrying pKSJ5 were labeled with [^{35}S]methionine, and the proteins they synthesized were immune precipitated with antibody specific for either gene III protein or colicin E3. The proteins were analyzed on sodium dodecyl sulfate-polyacrylamide slab gels. Minicells carrying pKSJ5 directed the synthesis of a new polypeptide which was, as expected, larger than the gene III protein and which reacted with antisera against both gene III protein and colicin E3 (labeled gIII-E3p) (Fig. 2). In addition, cells bearing pKSJ5 synthesized a smaller polypeptide (labeled E3 imm) that was immunoprecipitated by antiserum against purified E3 immunity protein as well as by the serum raised against native colicin E3, which is actually a heterodimer of colicin and immunity protein (22). Cells with pND372, in which gene III is cloned in pBR322, also synthesized a polypeptide which had the mobility expected for this gene III protein derivative and which was immunoprecipitated by antiserum against gene III protein but not by antiserum specific for colicin E3.

It should be noted (Fig. 2, lane g) that a small amount of unprocessed precursor gene III protein is present. On lower-percentage gels of these minicells (data not shown), a comparable amount of unprocessed fusion protein can be seen. Thus, even in minicells, where processing is relatively inefficient (1), most of the hybrid protein is processed for export.

Activity of gene III protein-colicin E3 fusions. Aliquots (20 μl) of IPTG-induced pKSJ5-bearing K1034 cell cultures or supernatants from induced cultures were spotted onto lawns of indicator cells (Fig. 3). The F$^+$ *btuB*$^+$ lawn of K38 can be infected by the two F-specific phages, f1 and f2, and killed by spots containing either colicin E2 or E3 (Fig. 3A, Table 2).

TABLE 2. Killing of *E. coli* by gene III-E3 fusion protein

Strain	Relevant genotype	Killing (or infection) by:					
		GeneIII protein-E3	f1	E3	E2	f2	E1
K38	F^+ *btuB*$^+$	+	+	+	+	+	+
K381	F^+ *btuB*	+	+	−	−	+	−
K1021	F^- *btuB*$^+$	−	−	+	+	−	+
K1017	F^+ *tolA*	−	−	−	−	+	−
K1041	F^+ *tolQ*	−	−	−	−	+	−
K1090	F^+ *tolQ*	−	−	−	−	+	−
K1089	F^+ *tolR*	−	−	−	−	+	−
K1042	F^+ *tolB*	−	+	−	−	+	+
K38(pKSJ17)	F^+ *imm*^{E3} *btuB*$^+$	−	+	−	+	+	+

Both cultures and supernatant medium from the fusion-producing strain exhibited definite, although turbid, killing spots on the F^+ *btuB*$^+$ lawn. No such effect was caused by cells expressing only the gene III protein derivative from the parental plasmid pND372. Although lawns of F^+ *btuB*, which lack the colicin receptor protein, were insensitive to colicin E3, they were sensitive to the gene III protein-colicin E3 hybrid (Fig. 3B). The colicin E3-sensitive F^- *btuB*$^+$ strain K1021 was not affected by the hybrid toxin (Table 2). Thus, the inhibitory effect of the gene III protein-colicin E3 hybrid is specifically directed at F^+ cells, regardless of the presence of wild-type BtuB protein. Every indicator strain tested was also checked for sensitivity to phages f1 and f2, to confirm the presence or absence of the F pilus, and for sensitivity to colicins E2 and E3, to check for the presence of the *btuB* receptor and thereby distinguish between tolerance (not killed by either E2 or E3) and immunity (sensitive to E2, immune to E3). The growth inhibition of the gene III protein-colicin E3 fusion was shown to be colicin E3 specific by testing it on a male strain that was immune to colicin E3. Growth of E3-immune K38(pKSJ17) was not inhibited by either the fusion protein or colicin E3, although it was sensitive to colicin E2. Just as E3 immunity protein blocks the killing action of natural colicin E3 (19), it can also block the action of this hybrid toxin. Thus, the growth-inhibiting activity of the gene III-colicin E3 fusion protein is absolutely dependent upon the presence of the F pilus and is a colicin E3-specific activity.

The activity of the fusion protein was also tested on F^+ strains with mutations in either *tolA*, *tolB*, *tolQ*, or *tolR* (Table 2). The presence of the F pilus on these mutants was verified by the fact that they could be infected by the male-specific phage f2, which attaches to the side, rather than the tip, of the pilus. Since *tolA*, *tolQ*, and *tolR* mutants cannot be killed by colicin E3 or infected by phage f1, it is not surprising that the growth of these mutants was not inhibited by the fusion protein. Interestingly, *tolB* mutants can be infected by phage f1 and are killed by colicin E1 but cannot be killed by colicin E2 or E3. *tolB* cells were not inhibited by the gene III-colicin E3 fusion protein.

The activity of the fusion protein was also detected by a method other than the spot test described previously. About 100 fusion protein-producing cells were spread on tryptone agar plates containing 1 mM IPTG and incubated for about 8 h at 37° C. Then, approximately 10^8 indicator cells in soft agar were overlayered, and the plates were incubated overnight. Cells that were sensitive to killing by the fusion protein gave rise to tiny turbid halos, like phage plaques, around microscopic colonies of fusion protein-producing cells.

Attempts to increase sensitivity to fusion protein. The killing spots made by the fusion protein on indicator lawns were always quite turbid compared with killing spots made by colicin-producing cultures. Furthermore, sonicating cultures of gene III protein-colicin E3 hybrid-producing cells did not result in quantitatively more killing activity than that observed from culture supernatants. Thus, export of the fusion protein appears to be efficient. This is consistent with the fact that most of the protein appears to be in the processed form, even in minicells (Fig. 2). Compared with those made by intact colicin, the spots made by the fusion protein corresponded to the turbidity of a spot made by about 10 to 20 ng of purified colicin E3 per ml or a 10^{-5} dilution of crude colicin produced by an induced culture. However, preparations of the gene III protein-colicin E3 fusion could be diluted as much as 10-fold before its activity was no longer detectable. Concentrating culture supernatants from induced fusion-producing cells about fivefold, by dialysis against dry Aquacide II-A, did not enhance the activity. It has been observed that W3110-derived strains are intrinsically 10- to 100-fold more sensitive to colicin E3 than K38-derived strains (K. Jakes, unpublished results). However, an appropriate W3110 derivative (K1074) was found to be no more sensitive to the fusion protein than any of the other strains. Thus, the turbid killing spots observed, such as those seen in Fig. 3, appear to be the maximum killing that can be effected by the gene III-colicin E3 fusion protein. Apparently, the efficiency of killing by this hybrid toxin is less than that of native colicin E3, which, in sufficient concentrations, can kill all of the cells exposed to it.

Release of the toxin from producing cells. Colicin E3 is synthesized as a soluble cytoplasmic protein. Its release from the producing cell depends upon the action of the colicin E3 lysis protein (23). Although pKSJ5 lacks the lysis protein gene, cells expressing the gene III-E3 fusion protein somehow released active toxin to the culture supernatant (Fig. 3). pND372, the parent to pKSJ5 (Fig. 1), expresses a gene III protein derivative that gets secreted to the cell exterior (11). This export is presumably initiated by the 18-residue-long amino-terminal signal peptide of gene III protein.

We have constructed a plasmid, pND277, which is identical to pKSJ5 except that the sequences encoding the gene III protein signal peptide have been deleted. Neither cultures nor culture medium from induced pND277-bearing cells affect the growth of the F^+ indicator. However, killing spots on indicator lawns were observed when the pND277-encoded fusion protein was mechanically released by sonication of producing cultures. Control sonicates without the colicin moiety did not result in killing spots. We conclude that the gene III protein signal peptide is required for export of the hybrid toxin. Apparently, in creating this gene fusion, we have altered not only the path of entry of the toxin into the target cell but also its mechanism of release from producing cells.

Requirement for active immunity protein in cells synthesizing gene III-colicin E3 fusion protein. Colicins are normally synthesized as cytoplasmic proteins, generally reaching a very high level in the cytoplasm before lysis protein action causes their release into the extracellular medium (21, 23). Immunity protein is also continuously synthesized by the colicinogenic cells to protect these cultures from the lethal effects of both the endogenous and exogenous colicin that they are producing. Colicins E2 and E3 are normally released from producing cells as heterodimers with their specific immunity proteins (22, 36). If, as was shown in the

TABLE 3. Lethality of *imm* geneIII-E3 fusion in *imm* cells

Transforming plasmid(s)	No. of Amp^r transformants from strain:			
	K561 F$^+$ *imm*	K561(pKSJ17) F$^+$ *imm*$^+$	K1034 F$^-$ *imm*	K1034(pKSJ17) F$^-$ *imm*$^+$
pKSJ15 + pKSJ17, *Kpn*I-digested	7[a]	2.4×10^4	3[b]	4.0×10^4
pKSJ5	7.2×10^4	5.7×10^4	2.7×10^3	5.6×10^4
pBR322	1.7×10^3	2.0×10^3	5.6×10^2	2.5×10^3

[a] All of these Amp^r transformants were also Kan^r.
[b] All of these Amp^r transformants were also immune to colicin E3.

preceding section, the export of the gene III-E3 hybrid protein is tightly coupled to its synthesis via the gene III protein signal sequence, then it may be that active toxin never accumulates in the cytoplasm of producing cells. In this case, immunity protein may be unnecessary for the survival of producing cultures which lack a receptor for the toxin to enter neighboring cells.

The gene for immunity protein on pKSJ5 was therefore inactivated by cutting the plasmid at its unique *Kpn*I site in the middle of the immunity gene (28), digesting with mung bean nuclease, reclosing the plasmid with ligase, and redigesting with *Kpn*I. The *imm* plasmid, pKSJ15, was isolated by transformation of strain K561 harboring the plasmid pKSJ17, which supplied the immunity function in *trans*. Only one of the resulting transformants synthesized active gene III-colicin E3 fusion protein, as determined by spot testing IPTG-induced cultures. Plasmid DNA from that transformant (a mixture of pKSJ17, which has a *Kpn*I site, and pKSJ15, which no longer has a *Kpn*I site) was digested with *Kpn*I, and equal amounts were used to transform both K561 and K1034 and those strains bearing pKSJ17. Transformants were plated on ampicillin-containing plates. As controls, undigested pKSJ5 DNA, which encodes both active gene III protein-E3 toxin and immunity protein, or pBR322, was transformed into both pairs of strains. The results are shown in Table 3. When the plasmid supplying active immunity protein (pKSJ17) was destroyed by *Kpn*I digestion, very few Amp^r colonies arose in K561 or K1034, while the control number arose in cells that contained a plasmid that encodes immunity protein. The Amp^r colonies in K561 were all screened and found to be also Kan^r; therefore, they carried intact pKSJ17 as well as pKSJ15 (without the *Kpn*I site). Strain K1034 carries a chromosomal kanamycin resistance gene, so Amp^r K1034 transformants could not be screened for the presence of pKSJ17 by testing for kanamycin resistance. However, when Amp^r transformants of K1034 were screened for immunity to colicin E3, they were all found to be immune and must therefore carry pKSJ17 also. It should be noted that the transformations were all performed by using uninduced strains (lacking IPTG) that carry the *lacI*^{q} allele; the level of synthesis of the hybrid gene III-colicin E3 protein should be very low in these cultures. Nevertheless, even though the colicin moiety of the fusion protein is probably inserted in the cell membrane as it is synthesized and a large pool of cytoplasmic toxin does not accumulate, the immunity protein is still essential for the cell. This was true both in the F$^+$ strain (K561), which had the possibility of being killed by toxin released from neighboring cells in the culture, and in the F$^-$ K1034 strain, which lacks the F-pilus receptor for the toxin.

DISCUSSION

We have constructed a plasmid encoding a hybrid protein in which the first 372 amino acids of the phage f1 gene III protein replace the amino-terminal two-thirds of the colicin E3 molecule. This hybrid protein uses the phage f1 receptor, the F pilus, in place of the normal BtuB protein colicin receptor to bind to cells and delivers the catalytic RNase activity of colicin to the cytoplasm of F$^+$ cells. In these hybrid proteins, the normal cell type specificity of colicin is altered; the fusion protein only kills F pili-containing bacteria. These results demonstrate that susceptibility to colicin is not absolutely dependent upon the presence of the BtuB colicin receptor. If an alternate means is provided for the enzymatic portion of colicin E3 to bind to the outer membrane, it is capable of killing those cells.

The fact that the hybrid toxin enters the cytoplasm of susceptible cells demonstrates that the phage f1 gene III protein, without accessory phage proteins or assembly into a phage particle, is sufficient for both pilus binding and initiating the entry of the colicin moiety into those cells. The colicin E3-specific activity thus serves as a marker for gene III protein function.

As is the case for both infecting phage f1 and native colicin E3, in order to cross the cell membranes after binding, the hybrid gene III-colicin E3 protein requires the wild-type form of the products of the *tolA*, *tolQ*, and *tolR* genes. Perhaps more surprising, the fusion protein also depends upon the TolB protein for activity. TolB is required for killing by colicin E3 but not for infection by phage f1. This result suggests that the hybrid gene III-colicin E3 protein may simply be using the F pilus to bind to the outer membrane. Pilus retraction brings the fusion protein to the cell membrane, and then the enzymatic portion of the colicin reaches the cytoplasm via the pathway normally used by colicin rather than the pathway used for f1, which does not involve the TolB protein. Alternatively, while the TolB protein may not be directly involved in the uptake of colicin, it is obviously required for some aspect of killing by the toxin.

It had been previously demonstrated that the receptor for colicin E3 could be bypassed by osmotically shocking *btuB* strains (41). However, the shock itself killed 95% of the cells, while osmotic shock in the presence of colicin gave 3 orders of magnitude more killing. Tilby et al. (41) also found that colicin-tolerant strains could not be killed, even with their rather drastic osmotic shock conditions. The *tol* mutants they isolated, however, were not mapped and therefore cannot be correlated with those used for this study.

Intact colicin E3 requires its amino-terminal domain for uptake or membrane translocation of the enzymatic activity into the cytoplasm (33). The glycine-rich region near the amino terminus of colicin E3 has been specifically implicated in having a role in colicin uptake (13). In the hybrid toxin, the amino-terminal two-thirds of the colicin molecule has been replaced by the f1 gene III protein. Perhaps, glycine-rich sequences of gene III protein (4) substitute for the glycine-

rich domain of the colicin to promote the entry of the colicin enzymatic activity into the cytoplasm.

The killing of susceptible cells by the fusion protein appears to be very inefficient compared with the killing by colicin E3. While the turbid spots made on indicator lawns may simply be due to the relatively low amount of fusion protein synthesized by induced producers, this does not seem to be the case. Killing activity from induced culture supernatants could be diluted about 10-fold, while concentrating culture supernatants from induced fusion-producing cultures did not enhance that killing activity. Thus the activity we observed appears to be a maximum. Since completely clear spots were never observed under any of the assay conditions we devised, it could be argued that the turbid spots made by the hybrid toxin on susceptible lawns are due to the inhibition of growth of cells in the lawn rather than outright killing of some fraction of those cells. However, the fact that strains that are immune to colicin E3 are insensitive to the hybrid toxin strongly suggests that this is a colicin-specific activity. Colicin E3 kills sensitive cells rather than inhibiting their growth, and turbid spots are observed when dilute solutions of colicin E3 are spotted on sensitive indicator lawns. One possible explanation for the weak killing activity is that sensitive *E. coli* cells have approximately 200 BtuB protein receptors (35), while F pili number at most a few per cell. Killing would therefore resemble that of very dilute colicin, with at most a few receptors per cell occupied by the toxin. It is also possible that the killing activity of the gene III-colicin E3 fusion protein is intrinsically less efficient than that of native colicin E3. This may be because normal processing, such as the cleavage of colicin that may occur as it traverses the bacterial membrane (24), is impaired by the removal of colicin sequences in the creation of the chimeric protein. Alternatively, it has been suggested that bound immunity protein and its timely removal play an active role in killing susceptible cells (22, 24, 36). This process also may be impaired in the fusion protein, and that may be responsible for the diminished killing activity of the fusion protein. We were unable to determine whether the secreted hybrid toxin contains bound immunity protein, since the amount of the hybrid protein found in the medium of labeled cells was too low to be detectable by antibody precipitation (K. Jakes and N. Davis, unpublished results). It is difficult to imagine how immunity protein would be secreted from these cells with the fusion toxin, since the toxin is secreted via the gene III protein signal sequence. The immunity protein is synthesized as a separate protein and does not carry its own signal sequence, nor do these cells carry the gene for the E3 lysis protein, which normally mediates the release of immunity protein into the medium of colicinogenic cells (23). Thus, in addition to having a cell surface receptor different from that of native colicin E3, the hybrid gene III protein-colicin E3 toxin leaves producing cells by a mechanism completely different from that of colicin E3. In spite of this altered mode of leaving the cell, the survival of cells carrying the gene for the hybrid colicin still depends upon their ability to synthesize the E3 immunity protein. Immunity protein was shown to be indispensable in cells carrying the gene for the hybrid toxin, even when its synthesis was not induced and producing cultures lacked receptors for the extracellular toxin.

The construction of the hybrid gene III-colicin E3 protein has also provided us with another means to examine the multiple functions of the phage f1 gene III protein. By using the fusion to the colicin moiety, the pilus-binding function of cloned gene III protein can be determined without the requirement that the protein be assembled in a phage particle. Both the pND372-derived fusion protein, which lacks the carboxy-terminal membrane anchor, and the pND277-derived fusion, which also lacks the gene III protein amino-terminal signal sequence, are capable of promoting the entry of the catalytic colicin E3 domain. Thus, the sequences deleted from gene III in these fusions are not required for the pilus-binding and membrane translocation functions of gene III. Other fusion protein constructions, using shorter segments of gene III protein, should further serve to define the specific sequences of the gene III protein that play a role in the binding and penetration of phage f1 into F pilus-containing cells.

The fact that the catalytic domain of colicin E3 can bind to bacteria via a structure (the F pilus) other than its normal BtuB protein receptor suggests that perhaps the colicin can be engineered to become an antibiotic with a broader range than it normally has. However, since phage f1 and colicin E3 share common entry requirements, in the *tolA*, *tolQ*, *tolR* pathway, that possibility remains to be established.

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